

AD_____

Award Number: W81XWH-08-1-0538

TITLE: Exploring Neurofibromin Function in a Yeast Model of NF1

PRINCIPAL INVESTIGATOR: Aaron Gitler, Ph.D.

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, PA 19104

REPORT DATE: September 2010

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-09-2010		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 SEP 2009 - 31 AUG 2010	
4. TITLE AND SUBTITLE Exploring Neurofibromin Function in a Yeast Model of NF1				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-08-1-0538	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Aaron Gitler, Ph.D. (PI) gitler@mail.med.upenn.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Pennsylvania Philadelphia, PA 19104-6058				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research And Material Command Fort Detrick, MD 21702-5012 Fort Detrick, MD 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT We hypothesized that the NF1 protein Neurofibromin has functions in addition to its Ras-GAP activity and proposed to develop a yeast NF1 model to define new functions as well as novel regulators of known functions. In year 1 of this project, we categorized genetic hits that we recovered from yeast screens. We divided hits into Ras-dependent and Ras-independent categories. In year 2 of this project, we have identified proteins that physically interact with IRA1 and IRA2 (the yeast homologs of human Neurofibromin). These data will facilitate future years of funding for this project, as we integrate the genetic and physical interaction data in order to get a better picture of the cellular pathways that Ira1 and Ira2 regulate, and then to validate if these interactions are relevant in animal models of the disease.					
15. SUBJECT TERMS NF1, yeast, IRA1, IRA2, ras, GTPase, TAP, purification, mass spectrometry, stationary phase, heat shock					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 10	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	10
Reportable Outcomes.....	10
Conclusion.....	10
References.....	10
Appendices.....	10

Introduction

Type 1 Neurofibromatosis (NF1) is a devastating human cancer syndrome, characterized by benign and malignant tumors of primarily neural crest origin. NF1 is caused by mutations in the NF1 gene, which encodes a large protein, called Neurofibromin. Neurofibromin is a large protein and has been shown to be able to function as a Ras-GTPase-activating protein (Ras GAP) to down-regulate ras signaling. Accordingly, NF1-deficient tumors show elevated ras signaling levels. Defining novel regulators of Neurofibromin's function will help to suggest therapeutic interventions. Because the Ras-GAP domain only comprises a small portion of the protein, we hypothesize that Neurofibromin has cellular functions in addition to its Ras-GAP activity. We have developed a yeast NF1 model to define NF1 disease mechanisms. Budding yeast, *Saccharomyces cerevisiae*, have two NF1-like genes, called IRA1 and IRA2.

In year one of the project, we generated *ira1* Δ and *ira2* Δ mutant cells and used these to perform genomewide genetic screens to identify, in an unbiased fashion, genes and pathways that interact functionally with Ira1 and Ira2 in yeast, and hopefully Neurofibromin in mammalian cells.

In year two of the project, to extend and complement the results from the genetic screens, we have begun isolating IRA1- and IRA2-interacting proteins. We have identified a list of 78 proteins that specifically interact with IRA2 and are continuing to identify IRA1-interacting proteins. These data will facilitate year 3 of this project as we continue to define the mechanism of action of these novel Ira1- and Ira2-interacting genes (year 1) and proteins (year 2).

Body

From the first year study of our project, we have defined a set of yeast genes that interact genetically with the *NF1* homologs, *IRA1* and *IRA2*, and have gone on to functionally categorize these genes as being either ras-dependent or ras-independent. One complication from these results is that we got a large number of hits that interacted genetically with *ira1* or *ira2* that, in the second year of our project, we have been devising strategies to efficiently narrow down the number of hits to those that are likely to be most relevant to human NF1. We reasoned that hits that also physically interact with IRA1 or IRA2 would be of particular value, since they could potentially serve as drug targets for manipulating the activity of NF1 or as buffers against the deleterious effects of NF1 mutation. Therefore, one major goal of the second year of our research project has been to identify IRA1 and IRA2 interacting proteins. The strategy we employed to identify IRA1 or IRA2 interacting proteins is coupling IRA1 or IRA2 purification with mass spectrometry identification of the co-purified proteins.

We have employed C-terminal tandem affinity purification (TAP) tag to facilitate the purification of IRA1 and IRA2, since TAP tag purification involves two steps purification which are based on different principles, and therefore can dramatically diminish the non-specific background and give us highly purified protein complexes.

So far, we have been optimizing experimental conditions to purify TAP tagged IRA1 and IRA2 using untagged wild type strains as a negative control. This purification procedure has been complicated by the fact that IRA1 and IRA2, like human NF1, are very large proteins. Both proteins contain more than 3000 amino acid residues and have a molecular weight more than 350 kDa. Further complications include the instability of the IRA proteins and their low expression level. We have been trying to avoid overexpressing IRA1 or IRA2 as that could lead to non-specific interactions irrelevant to the physical function of the IRA

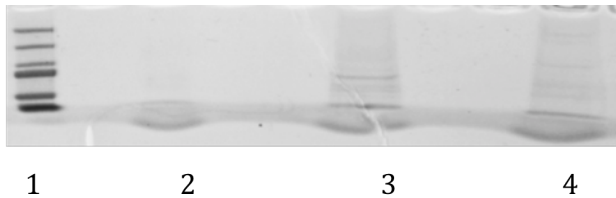


Figure 1. TAP-purification of IRA2-interacting proteins. Coomassie blue staining results of IRA2-TAP purification. Lane 1. Molecular weight Marker; Lane 2. WT control (stationary phase); Lane 3. IRA2-TAP (stationary phase); Lane 4. IRA2-TAP (heat shock treated)

proteins and complicate the subsequent data analysis and interpretation. Using a TAP-tagged IRA2 strain, we succeeded in improving the purification efficiency and optimizing the growth conditions. We reasoned that we should be able to optimize the IRA2 expression by growing cell under conditions where IRA2 was known to be important for yeast cells to grow normally

under those conditions. It has been known that IRA2 is important for yeast cells to enter stationary phase or for survival under heat shock condition. In our recent purification experiments, we used yeast cells grown to stationary phase or under heat shock conditions to purify TAP-tagged IRA2. We were able to increase the yield of the purification to a degree that we were able to see several specific protein bands on the SDS-PAGE gel when stained with Coomassie blue (Figure 1).

We isolated these bands from the gel and subjected them to mass spectrometry in order to identify the associated proteins. This resulted in the identification of a total 78 proteins from stationary phase and heat shock treated IRA2 samples (identified at least five-fold more peptide fragments in one of the TAP tagged samples than in the untagged WT control sample, Table 1. Importantly, the protein GPB1, which has been previously reported to physically associate with IRA2 was also recovered from heat treated samples, indicating that the purification and mass spectrometry are indeed successful. This suggests that some of the additional proteins that we identified, if their interactions are validated, will likely also be relevant to NF1 function.

It is worthwhile to mention that the interacting protein profile of IRA2 between two growth conditions are very different with some interactions present more abundant in stationary phase cells and some interactions occur mainly in heat shocked cells. This difference might be reflecting different function/regulation of IRA2 under these two growth conditions. It also highlights the necessity to identify protein interactions under various different experimental

conditions. We have tagged IRA2 and IRA1 with different tags and now are under the process of tagging the hits for Co-IP verifying the physical interactions identified from TAP purification. We are also now in the process of repeating the purification of IRA2 and trying to optimize the growth conditions for IRA1. Based on our experience with IRA2, we are confident that we will be able to rapidly optimize the procedure for isolating IRA1-associated proteins. It will be of great interest to determine those interacting proteins that are in common to IRA1 and IRA2 as well as those that are specific to either IRA1 or IRA2.

Table 1. Hits from IRA2-TAP purification and mass spectrometry. Number of peptide fragments recovered is listed for the control strain (WT) and the IRA2 strains subjected to either stationary phase or heat shock.

WT (control)	Ira2 (stationary phase)	Ira2 (heat shock)	Interacting Protein
7	137	71	SSA2
1	97	175	IRA2
1	89	161	IRA2
1	49	142	URA2
1	33	15	HSC82
1	31	6	HSP104
1	30	1	DED1
1	25	28	RPL4B
1	23	11	PFK2
2	22	11	KAR2
1	21	19	SSA1
1	21	1	SSA4
1	19	13	CDC19
1	18	7	RPS7b

1	17	23	PFK1
1	16	6	GRS1
1	16	1	TIM44
1	15	1	YEF3
1	15	4	HOR2 OR RHR2
1	14	7	RPS3
1	13	9	CDC19
1	13	5	PMA2
1	13	3	ILV5
1	12	9	RPS0b
1	12	6	RPL13b
1	10	21	FBA1
1	10	9	PSA1
2	10	7	RPS1a
1	9	8	RPS14b
1	9	6	SSC1
1	9	6	YGL245W
1	9	1	FAS1
1	8	9	RPL2b
1	8	7	ADE5,7
1	8	6	RPS24a
1	8	3	RPS20
1	8	1	RPG1
1	8	1	YHR020W

1	7	8	RPS13
1	7	8	RPS18a
1	7	2	PAB1
1	7	2	RPL6b
1	7	1	RPL9a
2	6	11	MDJ1
1	6	2	CCT8
1	6	2	RPL16a
1	6	1	UBP3
1	6	1	YEF3
1	5	23	ATP3
1	5	15	RPL3
1	5	7	RPS6b
1	5	6	ILV2
1	5	6	HSP104
1	5	5	RPS1b
1	5	3	TEF4
1	5	1	PBP1
1	5	1	RPS22
1	4	16	ATP1
1	4	8	ACC1
1	4	7	RPL18b
1	4	6	ADH1
1	4	6	RPS15
1	4	6	TUB2
1	4	6	RPT5
1	3	106	GPB1

1	3	12	MIR1
1	3	7	HSP42
1	1	12	IDP2
1	1	11	YAT2
1	1	10	ACS1
1	1	8	IRA1
1	1	7	GDH2
1	1	6	ATP2
1	1	6	PET9
1	1	6	RPL10
1	1	5	HSP60
1	1	5	RPL23a
1	1	5	RPL7b

Key Research Accomplishments

- Optimized protein purification procedure for IRA2.
- Identified a list of yeast proteins that interact physically with IRA2.

Reportable Outcomes

- Postdoctoral Fellow Xiaodong Fang, Ph.D., presented this work at the University of Pennsylvania School of Medicine Yeast Data Club Meeting.
- Results from the genetic screen and proteomics study will be presented at the American Society for Cell Biology (ASCB) meeting in Philadelphia in December, 2010.

Conclusion

In year 2 of our project we have built off of our results from the genetic screens during year 1 and have initiated a proteomics approach to identify yeast proteins that physically associate with NF1 homologs IRA1 and/or IRA2. We have developed experimental conditions to isolate IRA2-interacting proteins and have identified 78-specific proteins that interact with IRA2. We are currently optimizing conditions to isolate IRA1-interacting proteins. These data will facilitate our continued mechanistic experiments aimed at identifying novel functions for Neurofibromin as well as defining novel regulators of its known function as a regulator of the ras signaling pathway.

Now that we have identified a set of genetic and physical interactions, we will pursue approaches to integrate these data. It is our experience that combining genetic and physical interaction data is more powerful than relying on either one individually.

References

None

Appendices

None